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Seed dispersal by animals: exact identification of source trees with endocarp DNA microsatellites

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Abstract

A long-standing challenge in studies of seed dispersal by animal frugivores has been the characterization of the spatial relationships between dispersed seeds and the maternal plants, i.e. the seed shadow. The difficulties to track unambiguously the origin of frugivore-dispersed seeds in natural communities has been considered an unavoidable limitation of the research field and precluded a robust analysis of the direct consequences of zoochory. Here we report that the multilocus genotype at simple sequence repeat (SSR; microsatellite) loci of the woody endocarp, a tissue of maternal origin, provides an unequivocal genetic fingerprint of the source tree. By comparing the endocarp genotype against the complete set of genotypes of reproductive trees in the population, we could unambiguously identify the source tree for 82.1% of the seeds collected in seed traps and hypothesize that the remaining 17.9% of sampled seeds come from other populations. Identification of the source tree for *Prunus mahaleb* seeds dispersed by frugivores revealed a marked heterogeneity in the genetic composition of the seed rain in different microhabitats, with a range of 1–5 distinct maternal trees contributing seeds to a particular landscape patch. Within-population dispersal distances ranged between 0 and 316 m, with up to 62% of the seeds delivered within 15 m of the source trees. Long distance dispersal events, detected by the exclusion of all reproductive trees in the population, accounted for up to 17.9% of the seeds sampled. Our results indicate strong distance limitation of seed delivery combined with infrequent long-distance dispersal events, extreme heterogeneity in the landscape pattern of genetic makeup, and a marked mosaic of multiple parentage for the seeds delivered to a particular patch.

Keywords: avian frugivores, microsatellites, seed dispersal, seed endocarp, seed shadow, spatial variation

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Introduction

For animal-dispersed, endozoochorous, plant species a seed shadow is the population recruitment surface (Janzen 1970; Connell 1971), the spatial pattern of seed distribution over the landscape relative to parent trees and other conspecifics that results from the process of seed dispersal. Frugivores determine the landscape pattern of seed distribution and density over available microhabitats and thus can have a dramatic effect on both the demography and genetic make-up of animal-dispersed plant species. The difficulties in understanding, measuring and analysing dispersal of seeds in natural communities has been considered as an unavoidable limitation of the research field (Wheelwright & Orians 1982), and the methodological shortcomings of tracking the origin of frugivore-dispersed seeds has precluded a deep and robust analysis of the direct consequences of zoochory (Levey & Sargent 2000).

Seed and pollen are the two vectors of gene flow in plants. Levels of gene flow have traditionally been assessed through indirect methods that infer average historical values from the extent of subpopulation differentiation under a particular model (Neigel 1997; Sork *et al*. 1999). Within population gene movement has been addressed by analysing the genetic structuring at a microgeographic scale

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(Alvarez-Buylla *et al*. 1996; Epperson & Alvarez-Buylla 1997; Aldrich *et al*. 1998; see also Ueno *et al*. 2000). A prevailing pattern in these studies is strong clumping of dispersed seeds, significant autocorrelation values over short distances, and extreme context-sensitivity of the pattern of genetic structuring depending on the particular landscape setting. More recently, the availability of hypervariable genetic markers (Tautz 1989) has allowed the direct assessment of contemporary gene flow in a landscape context, based on parentage exclusion analyses (Luikart & England 1999). Direct analyses in plants have concentrated on paternity assessment for progeny arrays of known maternity with two main objectives: analysis of male fertility (Meagher 1986, 1991), and estimation of pollen-mediated gene flow (see e.g. Devlin *et al*. 1988; Devlin & Ellstrand 1990). However, very few papers so far have applied a direct approach to the study of seed dispersal, the second component of gene flow. This is in part due to the limited resolution of parental exclusion methods, especially when the two parents are unknown, and the impossibility of distinguishing the father and mother in cosexual species (such as hermaphrodites or monoecious; Dow & Ashley 1996; Schnabel *et al*. 1998a). Cytoplasmic markers could also be used to exclude maternity but they usually lack enough variation for individual maternity assignments (Ouborg *et al*. 1999).

Here we report a new approach for the identification of the source tree of individual *Prunus mahaleb* seeds recovered from animal frugivores (either defecated or regurgitated) based on analysis of simple sequence repeats (SSRs or microsatellites) (Tautz 1989) from the seed endocarp DNA. The multilocus genotype of the seed endocarp unambiguously identifies the maternal tree of the seed due to the fact that the endocarp tissue in *Prunus* species, as in most drupaceous species, is of maternal origin, derived from the carpelar wall (Roth 1977). We determined the minimum number of female trees contributing progeny to specific locations in the landscape and the fraction of own progeny dispersed beneath a given source tree relative to progeny from other neighbouring trees. In addition, we analysed the spatial relationships between individual dispersed seeds and their source tree by determining seed dispersal distances directly.

Materials and methods

Study site and field methods

This study was conducted in the Reserva de Navahondona-Guadahornillos (Parque Natural de las Sierras de Cazorla, Segura y las Villas, Jaén province, southeastern Spain) in a *Prunus mahaleb* population located in Nava de las Correhuelas (1615 m elevation) (Valle *et al*. 1989). Frugivorous birds and mammals visiting *P. mahaleb* trees in Spanish populations usually behave as legitimate seed dispersers swallowing the fruits whole and defecating and/or regurgitating the seeds, usually after leaving the tree (Jordano 1994). Most seed rain of *P. mahaleb* in the study areas is contributed by frugivorous birds. Seed rain and the resulting recruitment pattern of seedlings and saplings are highly patchy, and largely restricted to microhabitats beneath woody cover in the vicinity of fruiting trees (Jordano & Schupp 2000).

Sampling strategy

Leaf tissue was sampled for total of 180 adult trees, representing 100% of the reproductive population, during the years 1996–2000 (Jordano & Godoy 2000). To compare the maternal genotype obtained from leaf tissue with the genotype of the seed endocarp, we sampled 1–3 seeds from the branches of four trees. In addition, we obtained the endocarp and embryo tissues from 11 seeds among the progeny (five families) obtained in diallel crosses from a concurrent study (P. Jordano, personal observation); thus, for these seeds both the sire and dam trees were known.

Naturally dispersed seeds were sampled in the dispersal season of 1996 with 20 replicate sets of two seed traps located in four different microhabitat types in the forest: beneath five focal *P. mahaleb* trees, beneath 10 mid-height shrubs (e.g. *Crataegus mongyna*, *Lonicera arborea*), beneath three pine trees, and beneath two pine trees with juniper understory. These replicate sampling points were a stratified random sample of a total of 481 sampling points used in a concurrent study of seed rain patterns. The types of microhabitats were a subset of those defined in Jordano & Schupp (2000). The adult reproductive trees in the population were mapped and their locations recorded in a GIS database, which also included the locations of the sampling sites with seed traps. A total of 95 seeds were randomly subsampled from those recovered from the traps, either in defecations or regurgitations of bird and mammal frugivores.

DNA extraction and microsatellite genotyping

DNA was extracted from 100 to 200 mg of fresh leaf tissue, sampled in 1998–99, using the rapid miniprep method of Cheung *et al*. (1993). Briefly, tissue was homogenized in 320 μL of extraction buffer (200 mM Tris-HCl pH 8.0, 70 mM EDTA, 2 M NaCl, 20 mM sodium bisulfite) with an electric drill (560 W; full speed) with attached plastic disposable pestles. After homogenization 80 μL of 5% sarcosyl was added and the sample was incubated at 65 °C for 30 min and centrifuged at 16 000 ***g*** for 15 min to remove insoluble material. DNA was precipitated by the addition of 90 μL of 10 M ammonium acetate and 200 μL of isopropanol. The mixture was incubated at room temperature for 5 min and centrifuged for 15 min at 16 000 ***g***. The pellet was washed with 70% ethanol, dried and resuspended in 100 μL TE buffer.

The seed endocarp was split open and separated by hand from the seed content (embryo plus vestigial endosperm), and collected for later grinding. We do not expect contamination of the endocarp tissue with remains of the seed content, as the whole embryo is separated from the endocarp by a thin seed coat that is cleanly extracted when the endocarp is split open. For the DNA extraction from seed endocarps we used the following modifications of the protocol for leaves: tissue was previously grinded in a mixer-mill and homogenized in 320 µL of extraction buffer, and the DNA finally resuspended in 50 µL TLE (200 mM Tris-HCl pH 8.0, 70 mM EDTA). DNA yields were estimated fluorometrically for leaf extracts, but they were too low (range 1–5 ng/µL) to be accurately estimated for endocarp extracts. For the endocarps we empirically tested a series of extract volumes in amplification reactions of microsatellite marker and found that 5 µL (1/10) of endocarp extract yielded consistent amplifications.

We tested a total of 43 primers pairs designed for cultivated *Prunus* and *Malus* species (A. Abbott 1998, personal communication; G. King 1998, personal communication; Cipriani *et al*. 1999; Downey & Iezzoni 2000; Sosinski *et al*. 2000) and selected a subset of nine markers that showed polymorphism in *P. mahaleb* for use in this study (Table 1).

As a template for the polymerase chain reaction (PCR) we used 30 ng of leaf DNA or 5 µL (1/10) of endocarp extract. PCR was performed in a final volume of 20 µL containing 67 mM Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.01% Tween-20, 0.01% BSA, 0.25 mM of each dNTP, 0.25 µM of each primer, and 0.5 U of *Taq* DNA polymerase. Reactions were incubated in a MJ Research PTC-100 thermocycler programmed for a 'touchdown' PCR as follows: an initial denaturation step at 94 °C for 2 min; 17 cycles of 92 °C for 30 s, annealing at 66–50 °C for 30 s (1 °C decrease in each cycle), and extension at 72 °C for 30 s; 19 cycles of 92 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. A final extension was programmed at 72 °C for 5 min. Amplified fragments were analysed using an ABI 377 Genetic Analyser (Applied Biosystems).

To assess the genotypic relationships among the leaf tissue, the endocarp of the seed, and the embryo, progeny from a diallel cross of known sires and dams were obtained in 1992 and 1993 and independent DNA extractions and genotypes were obtained from each tissue. When comparing embryo to parental genotypes, a strict Mendelian inheritance was observed with no evidence for the occurrence of null alleles.

Source tree identification

For the study of seed dispersal, a total of 182 adult trees and 95 dispersed seed endocarps were genotyped. The source tree for individual dispersed seeds was identified by comparing the endocarp multilocus genotype with the complete set of genotypes of reproductive trees in the population. For each sampled seed, the adult individual having a genotype matching the seed endocarp genotype was assigned as the mother tree. Matches between endocarp and adult genotypes were found by examining all possible pairwise comparisons between endocarps and adult trees. A hypothesis of identity ($r_p = 1$, $r_m = 1$) was tested with program KINSHIP (version 5.0.5) and the significance estimated by a jackknife resampling method (Queller & Goodnight 1989). Briefly, a hypothesis about pedigree identity between an endocarp and a tree (leaf tissue) is defined in terms of r_p and r_m, the probabilities that individuals in the pair share an allele by direct descent from their father or mother, respectively. For the hypothesized relationship of full genotypic matching, both *r*-values would be 1.0 (K.F. Goodnight, personal communication). Given the hypothesis, the *r*-values, the population allele frequencies, and the two genotypes under consideration are used to estimate the likelihood that this genotype combination could have been produced by the relationship as specified. The method calculates a likelihood for two such hypotheses, the primary hypothesis (identity) and a null hypothesis (no identity), and the ratio between them (primary/null). A high value of the ratio favours the primary hypothesis and a low value rejects it in favour of the null hypothesis. The significance level of a given ratio is estimated empirically, by simulation, generating pairs of individuals using the hypothesis settings and the allele frequencies and determines the ratio needed to reject the null hypothesis with a given $P < 0.05$ (Queller & Goodnight 1989).

Results

Genotyping of seed endocarp tissue for the identification of the source (maternal) tree

We successfully extracted DNA from the woody endocarp tissue of individual *Prunus* seeds and complete microsatellite genotypes were thus obtained. Reliable genotypes were obtained from endocarp tissue, both from freshly collected seeds and from seeds collected in seed traps. Most seeds sampled in seed traps were dispersed by birds, small to medium-sized passerines, with short gut passage times that either defecate or regurgitate them (Jordano & Schupp 2000). In addition, we have also amplified successfully extracted DNA from endocarps of seeds dispersed by red foxes (*Vulpes vulpes*), stone martens (*Martes foina*) and large birds such as wood pigeon (*Columba palumbus*) and carrion crow (*Corvus corone*). Thus, the miniprep DNA extraction protocol can be used to obtain adequate amounts of DNA even from seeds which have passed the digestive tract of frugivores.

Because endocarp tissue is of maternal origin its multilocus genotype should be identical to that of the mother

Table 1 Multilocus genotypes for leaf, endocarp, and embryos of *Prunus mahaleb* determined from scoring of simple sequence repeat (SSR) loci. For each maternal tree, the presence of alleles for each of nine SSR loci is given for the leaf tissue and for seeds of its progeny. Two or three seeds were selected from each tree and the allelic profile for the SSR loci is given for both the endocarp tissue (labelled with T) and the embryo (labelled E). Empty boxes indicate those instances (all correspond to embryos) where a particular allele is not present in the mother tree. Endocarps have in all instances identical multilocus genotypes to the leaf tissue. For example, embryo 1926 5E is homozygous for locus pchgms3 (191/191), while both the leaf tissue (1926) and the endocarp tissue (1926 5T) are heterozygous for this locus (179/191). Alleles are designed by the size (bp) of their products

		TREE 1926					TREE 1927				
			Endocarps		Embryos			Endocarps		Embryos	
Locus	Allele	Leaves 1926	1926 5T	1926 6T	1926 5E	1926 6E	Leaves 1927	1927 1T	1927 4T	1927 1E	1927 4E
UDP96-001*	114										
	122									□	
	124	■■	■■	■■	■■	■■	■■	■■	■■	■	■■
pchgms3†	179	■	■	■		■	■■	■■	■■	■■	■■
	191	■	■	■	□■	■					
UDP96-018*	246	■■	■■	■■	■■	■■	■	■	■	■□	
	248						■	■	■		■□
UDP97-403*	105						■	■	■		■
	107	■■	■■	■■	■■	■■	■	■	■		
	109										□
PS12A02†	171										□
	175	■	■	■		■□					
	185	■	■	■	■□		■■	■■	■■	■■	■
pchcms5†	235	■■	■■	■■	■■	■	■	■	■	■	
	239					□	■	■	■	■	
UDP98-406*	98	■	■	■	■	■	■■	■■	■■	■	■■
	102	■	■	■	□	■				□	
UDP97-402*	140										□
	148	■	■	■	■□	■	■■	■■	■■	■■	■
	152	■	■	■		■					
MS01A05‡	196						■				
	200	■■			■■	■■	■				

		TREE 1928							TREE 1847				
			Endocarps			Embryos				Endocarps		Embryos	
Locus	Allele	Leaves 1928	1928 1T	1928 3T	1928 4T	1928 1E	1928 3E	1928 4E	Leaves 1847	1847 2T	1847 3T	1847 2E	1847 3E
UDP96-001*	122	■	■	■	■								□
	124	■	■	■	■								
	126								■■	■■	■■		■
pchgms3†	179	■	■	■	■	■□	■	■				□	□
	187								■	■	■		■
	195	■	■	■	■		■	■	■	■	■	■	
UDP96-018*	248	■■	■■	■■	■■	■■	■■	■■	■■	■■	■■	■■	■■
UDP97-403*	107								■	■	■	■	
	109											□	
	125	■	■	■	■	■	■	■	■	■	■		■□
	145	■	■	■	■	■	■	■					
PS12A02†	185	■■	■■	■■	■■	■■	■■	■■	■■	■■	■■	■	■■
	189											□	
pchcms5†	235	■■				■■	■■	■■				□	□
	239								■■	■■	■■	■	■
UDP98-406*	98	■	■	■	■	■	■	■					□
	100	■	■	■	■	■	■	■	■■	■■	■■	■■	■
UDP97-402*	142	■		■					■■	■■	■■		■
	148	■		■			■□						□
MS01A05‡	200	■											
	202	■							■■	■■	■■		

*Cipriani *et al*. (1999); †Sosinski *et al*. (2000); ‡G. King, personal communication.

tree. We confirmed this for individual mother trees by comparing the genotypes of leaf and tissue from their seed progeny (both endocarp and embryo) (Table 1). We further tested this hypothesis by analysing 1–5 seeds per family obtained in a series of diallel crosses; for each of these seeds both the embryo and the endocarp were genotyped and compared to those of the known dam and sire trees (data not shown). As expected, the embryo genotypes were variable and compatible with the genotypes of the known sire and dam trees. On the other hand, the endocarp genotypes of seeds collected from the same tree were identical to each other and to the genotypes obtained from the leaf tissue of their corresponding maternal trees (Table 1). The genotyping of seed endocarp thus provides a convenient tool for the unambiguous assignment of maternity for seeds dispersed by frugivorous animals.

Identification of the maternal source trees for dispersed seeds

We genotyped a total of 182 reproductive trees, ~100% of the potential maternal trees for the 1996 seed crop, and 95 dispersed seeds collected in seed traps in different microhabitats. In this population we determined a total of 182 distinct multilocus genotypes, each adult tree in the population showing a unique multilocus genotype. Gene diversity was 0.996 ± 0.001 (Nei 1987), with mean expected heterozygosity of 0.586 and an average of 6.7 alleles per locus (estimated with the ARLEQUIN package, version 2.0; Scheiner *et al*. 2000). For 78 seeds in the seed traps (82.1%) a significant genotype match was found with a single adult tree in the population. In these cases, the matching tree was identified as the maternal source tree of the seed. No matching genotypes were found among adult trees in the population for the remaining 17 seeds, indicating that they come from maternal trees located in other populations. This result reveals the occurrence of long-distance interpopulation seed dispersal in this system.

Analysis of the seed shadow

A typical seed shadow of *Prunus mahaleb* in our study area consists of most seeds dispersed beneath the canopy of the maternal trees or beneath cover of mid-height shrubs (*Crataegus monogyna*, *Lonicera arborea*, *Rosa* spp.), with much lower seed fall figures beneath low shrubs (*Juniperus* spp., *Berberis vulgaris*) or pine trees.

The identification of the source tree for individual dispersed seeds allowed us to characterize the composition and structure of the seed shadow in the population. Different microhabitats within the landscape were sampled for dispersed seeds and their tree of origin was identified. The mean number of maternal trees contributing progeny to any seed sampling location ranged between 1 and 5 (Table 2), and this number differed significantly among microhabitat types ($F = 4.78$, $P = 0.007$, d.f. = 3, 34). Sampling sites beneath *Prunus* and mid-height shrubs had a greater number of maternal trees contributing seed than sites beneath pine trees (Scheffé a posteriori test, $P < 0.04$ for both comparisons).

Most of the seeds dispersed beneath a *P. mahaleb* tree were from its own progeny (Table 2, Fig. 1), although trees differed in the proportion of their own progeny represented in the seed rain beneath them. Thus, individual seeds dispersed beneath fruiting trees originated from a varied number of conspecifics. The sampling points located beneath mid-height shrubs close to a *P. mahaleb* tree typically showed a large proportion of the seed shadow composed by progeny from the nearest tree (the 'focal' tree; Fig. 1). The relative representation of progeny from the 'focal' tree dramatically decreased with increasing distance, and was very low beneath pine trees growing at the forest edge, away from the shrub patches where most *P. mahaleb* source trees are located. This indicates not only a potentially strong distance limitation, but also extreme clumping or aggregation of different seed genotypes.

Direct estimation of dispersal distances

The fact that both the maternal trees (seed sources) and the sampling points with seed traps were georeferenced allowed a direct estimation of seed dispersal distances, even with this limited sample of dispersed seed (Fig. 2). As expected from observational study of flight patterns and frugivore foraging (Jordano & Schupp 2000), most dispersal distances were short, within 10 m of the maternal source tree. The median dispersal distance for this seed sample was 6.1 m (0.0–46.1 m) (median and 25–75% percentiles), with an extremely skewed frequency distribution (Fig. 2), and approximately 65% of the dispersal distances being < 20 m. These empirically derived estimates correlate well with our previous characterization of the seed shadow based on direct watches of bird foraging (Jordano & Schupp 2000). Interestingly, the dispersal distances estimated for seeds trapped in different microhabitats differed significantly ($H = 32.81$, $P < 0.0001$; Kruskall–Wallis test). Seeds dispersed beneath pine trees had longer dispersal distances than those sampled beneath *P. mahaleb* trees, mid-height shrubs, or pine trees with low shrub understory.

Discussion

The endocarp tissue from *Prunus mahaleb* seeds dispersed by frugivores, that either regurgitate or defecate them, can be used to identify the maternal source tree when their genotypes are compared with genotypes of adult trees in a

Table 2 Assignment of maternal trees for animal-dispersed, *Prunus mahaleb* seeds sampled in seed traps

Seed sampling point*	Focal tree*	Other trees contributing seeds to sampling point*	Number of identified trees contributing seeds	Number of seeds with maternity unassigned	Estimated number of trees contributing seeds†
Beneath *Prunus mahaleb*					
795	372^{5}	1928^{1}	2	1	3
793	1927^{3}	1846^{2} 1837^{1}, 1921^{1}	4	0	4
799	399^{6}	—	1	2	3
792	1843^{6}	—	1	0	1
791	1921^{6}	1823^{1}, 1834^{1}	3	1	4
Beneath mid-height shrubs					
830	1927^{0}	372^{1}, 364^{1}, 370^{1}	3	2	5
831	1927^{1}	396^{3}, 1925^{1}, 1924^{1}, 397^{1}	5	0	5
832	1927^{0}	1849^{1}	1	4	4
833	1927^{0}	379^{1}, 383^{1}	2	0	2
826a	1921^{4}	1925^{1}	2	1	3
826b	1843^{1}	—	1	0	1
827	—	1923^{2}, 1164^{1}, 1831^{1}	3	3	5
828	—	361^{1}, 362^{1}, 1927^{1}, 396^{1}, 1845^{1}	5	0	5
837	399^{3}	1927^{2}	2	1	3
838	399^{3}	1927^{1}	2	1	3
Beneath pine trees					
866	399^{0}	1939^{1}, 364^{1}	2	0	2
867	399^{0}	1848^{1}, 1188^{1}	2	0	2
846	399^{0}	1932^{1}	1	0	1
847	399^{0}	1190^{1}	1	1	2
849	399^{0}	1179^{1}	1	0	1

*Numbers refer to the codes of the sampling points or the tree codes. Superscripts indicate the number of seeds that were assigned to each maternal tree. Each of the 'focal' trees had a set of seed traps associated.
†Figures include the number of identified maternal trees plus the estimated number of trees contributing the unassigned seeds. Thus, whenever two unassigned seeds had mismatching genotypes we computed two distinct (unassigned) maternal trees.

population. This is expected on the basis of the anatomical origin of *Prunus* drupes, with a fleshy mesocarp and a woody endocarp derived from the diploid carpelar tissues of the mother flower (Roth 1977). For dispersed seeds that still retain pieces of pulp, the maternal source tree could also be identified by genotyping pulp remains. However, using seeds is preferable as the pulp rapidly molds and disappears from fruit remains sampled with seed traps. Thus, this method of direct genotyping can be easily combined with regular sampling schemes of seed rain using seed traps (Kollmann & Goetze 1997; Harms *et al*. 2000) to assess patterns of seed dispersal at the landscape level. Estimating seed dispersal distances based on seed traps can be effective depending on the robustness of the seed trap sampling scheme. Ideally, seed traps should be arranged randomly throughout the habitat, in a stratified scheme according to major landscape units, and then a sufficient number of the seeds sampled in the traps should be selected for genotyping. We are working with such an intensive sampling scheme, with a network of 1400 seed traps in two populations, to assess the robustness and potential biases of direct estimates of dispersal distances (J.L. García-Castaño, J.A. Godoy & P. Jordano, in preparation).

The approach we describe here can be applied to a variety of endozoochorous seed species that typically show a thick endocarp, although care should be taken in determining the anatomical origin of the tissue analysed. When assessing species with complex fruit structures, such as arillate seeds, etc., a preliminary comparison with other maternal tissues can be undertaken to assure the reliability of using a particular tissue to compare with the maternal genotype. The method can also be used with wind-dispersed species that typically show ancillary structures such as wings, pappus, etc., presumably of maternal origin. Our approach can be used to estimate relative female fertilities, the diversity of trees contributing seeds to particular landscape patches and thus, the heterogeneity of the seed rain over the landscape, especially in relatively small populations. Some of these parameters can be estimated even with incomplete genotyping of the adults in larger populations (Slate *et al*. 2000). For instance, an exclusion approach requiring limited genotyping effort can be used to test whether a subset of candidate trees in

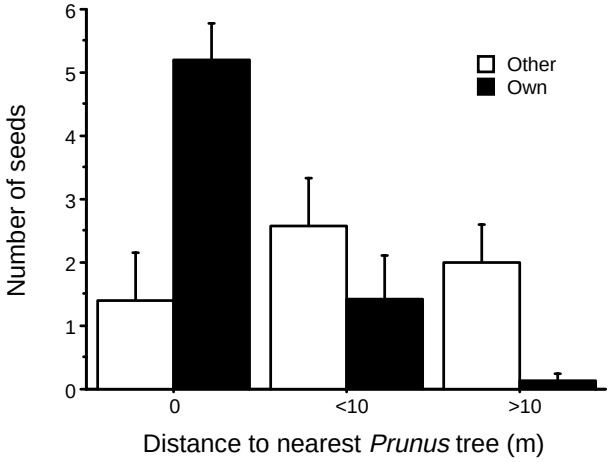

Fig. 1 Type of progeny of animal-dispersed *Prunus mahaleb* seeds sampled in seed traps as a function of distance to the nearest *P. mahaleb* tree. 'Own' refers to seeds from the nearest tree, i.e. closest to the seed trap locations in the forest. 'Other' refers to those seeds whose maternal tree was not the nearest tree.

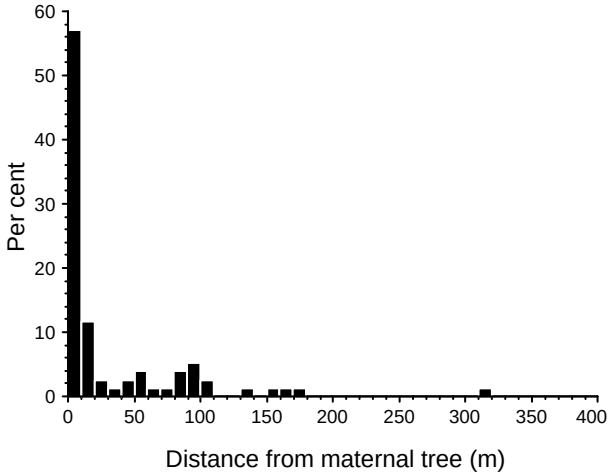

Fig. 2 Frequency distribution of seed dispersal distances estimated by direct genotyping of seed endocarp of animal-dispersed *Prunus mahaleb* seeds sampled in seed traps.

the neighbourhood of a given seed sampling point are the source for the sampled seeds.

Sampling points beneath *P. mahaleb*, mid-height shrubs, or pines with low shrub understory recruited seed rain from a larger number of parents than points beneath pines. Thus, those patches in the habitat with dense vegetation cover not only recruited larger numbers of seeds (Jordano & Schupp 2000) but also showed greater small-scale genetic diversity (estimated from the number of distinct maternal trees contributing progeny). The fact that *P. mahaleb* trees typically grow associated to patches with high shrub cover results in each maternal tree acting as a dispersal site, or 'sink', for seeds dispersed from other conspecifics. A heterogeneous landscape, with a marked mosaic of covered and open microhabitats, and the nonrandom movements of frugivores strongly selecting covered microhabitats (Kollmann & Schneider 1996; Jordano & Schupp 2000) result in a dramatic heterogeneity in the provenance of the seeds and a marked clumping of the distinct seed genotypes at different sites over the landscape. This has important implications in conservation and management of forest species: simply adding a low shrub cover beneath pine trees would increase significantly both the density of dispersed seeds and its genetic heterogeneity. Whether or not this translates into actual recruitment of seedlings and saplings would depend on factors acting during the postdispersal stages of regeneration.

Our empirically derived estimates of dispersal distances reveal an extremely skewed frequency distribution, with most seeds dispersed in the neighbourhood of the maternal tree. These estimates, relying on seed trap sampling, do not account for the fact that secondary dispersal after delivery by frugivores might occur. This is not a major influence in our system, as secondary dispersal by ants is infrequent (P. Jordano, personal observation), although seed caching by *Apodemus sylvaticus* has been reported (Hulme 1997; E.W. Schupp and P. Jordano, personal observation).

Despite the fact that no less than 60% of seed dispersal events were within 20 m of the maternal source tree, we recorded ~10% of dispersal distances > 100 m away. These were consistently seeds dispersed beneath pine trees, generally located along the forest edge surrounding the deep soil 'poldje' patches and lower rocky slopes where *P. mahaleb* trees grow. Thus, any *P. mahaleb* tree has a relatively long distance to pine trees in this population. We have previously reported (Jordano & Schupp 2000) that the seed rain to pine trees is largely contributed in our study area by *Turdus viscivorus*, a thrush species that typically flies to perch on pine trees after feeding on *P. mahaleb*. In contrast, the seed rain reaching covered microhabitats (beneath *P. mahaleb*, mid-height shrubs and patches with low shrubs) is contributed by an assortment of up to six small-bodied frugivore species (warblers, robin, and redstarts) with short flight distances. Therefore our results, in combination with previous field observations, indicate that most long-distance dispersal in this population is directed to a particular landscape patch despite a relatively diverse frugivore assemblage; and those long-distance dispersal events are contributed by a single disperser species.

All our estimates of dispersal distances do not include the very long dispersal distances from other populations (immigrant seeds), so they should be taken as a within-population dispersal estimate. At least 17 sampled seeds (17.9%) were not assignable to any adult maternal tree in

the population and this can be taken as an upper estimate of the fraction of immigrant seeds in the seed rain. This result reveals the occurrence of long-distance interpopulation seed dispersal in *P. mahaleb*, presumably from surrounding populations which are 3–5 km away. Evidence for extensive gene flow in the fragmented *P. mahaleb* populations has been reported, with high levels of within-population genetic diversity estimated from random amplified polymorphic DNA (RAPD) markers (Jordano & Godoy 2000). We are currently working with material from the two nearest populations to be able to identify the source population for unassigned seeds.

Dispersal distances estimations add evidence to our previous finding of a strong spatial autocorrelation in the 0–20 m distance for the coancestry estimates among adult trees in this population based on SSR and RAPD markers (Jordano & Godoy 2001). Despite the fact that there are a number of multiple postdispersal influences determining survival of seeds and seedlings to adulthood that can erase the initial spatial pattern determined by frugivore activity (Jordano & Herrera 1995; Rey & Alcántara 2000), it seems that patterns of genetic structuring in this population track the influences of seed delivery by frugivores. This finding also reveals that fragmentation of populations of animal-dispersed woody species can result in severe genetic bottlenecks by isolating patches of genetically similar adult trees (Loiselle *et al*. 1995; Nason & Hamrick 1997; Schnabel *et al*. 1998b). Also, the extinction or marked seasonal or annual changes in certain frugivore species can severely alter the spatial patterns of genetic make-up in the seed shadow by truncating and/or redirecting gene flow via seeds.

We suggest that a distance limited, spatially aggregated pattern in the genetic make up of the seed shadow, as reported here for *P. mahaleb*, is a typical situation of many animal-dispersed, fleshy-fruited plant species that deserves further study. Our analysis also indicates that relatively long dispersal distances are probably not infrequent, but represent an extremely small fraction of the dispersal events in a given reproductive episode, and are extremely directed in the heterogeneous landscape, as the result of nonrandom foraging by a limited set of species in the disperser assemblage.

The combined application of molecular tools based on hypervariable SSR markers and detailed observational and experimental ecological data has allowed a robust characterization of the influence of frugivore foraging on *P. mahaleb* population dynamics. This pervades the demographic effects — frequently the main and unique subject of ecological studies — to show up as a multiple, combined, demographic and genetic influence. To our knowledge this is the first study to combine detailed ecological and molecular approaches to reveal the origin and spatial patterns of the genotypes of animal-dispersed seeds. Here we have reported the results of a limited sampling design to reveal the potentials and weaknesses of our approach, and more in depth study is under way. The combination of carefully designed ecological experiments and sound laboratory analyses with molecular markers pave a promising avenue for future studies of plant–frugivore mutualisms.

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These results are part of a broader study on the effect of animal frugivores on the genetics and demography of fleshy-fruited plants. Ongoing research includes the use of microsatellite markers in parentage analysis to study seed dispersal in relation to frugivore foraging. P. Jordano is interested in the evolutionary ecology of plant-animal mutualisms and the demographic and genetic effects of the interaction. J. A. Godoy is interested in the development and application of molecular techniques to studies of ecology and conservation of endangered species (individual, parentage, and population genetic analyses). They coordinate the Molecular Ecology Laboratory at the Estación Biológica de Doñana.
